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Manipulation of activity and orientation of membrane-reconstituted di-tripeptide transport protein DtpT of Lactococcus lactis

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Summary

The di-tripeptide transport system (DtpT) of Lactococcus lactis was purified to apparent homogeneity by pre-extraction of crude membrane vesicles with octaethylene glycol monodecyl ether ($C_{10}E_8$), followed by solubilization with n-dodecyl-β-D-maltoside (DDM) and chromatography on a Ni-NTA resin. The DtpT protein was reconstituted into detergent-destabilized preformed liposomes prepared from E. coli phospholipid/phosphatidylcholine. A variety of detergents were tested for their ability to mediate the membrane reconstitution of DtpT and their effectiveness to yield proteoliposomes with a high transport activity. The highest activities were obtained with TX_{100}, $C_{12}E_8$ and DM, whereas DDM yielded relatively poor activities, in particular when this detergent was used at concentrations beyond the onset of solubilization of the preformed liposomes. Parallel with the low activity, significant losses of lipid were observed when the reconstitution was performed at high DDM concentrations. This explained at least part of the reduced transport activity as the DtpT protein was highly dependent on the final lipid-to-protein ratios in the proteoliposomes. Consistent with the difference in mechanism of DDM- and TX_{100}-mediated membrane protein reconstitution, the orientation of the DtpT protein in the membrane was random with DDM and inside-in when TX_{100} was used. The methodology to determine the orientation of membrane-reconstituted proteins from the accessibility of cysteines for thiol-specific reagents is critically evaluated.

Keywords: purification, reconstitution, membrane protein, transport, di-tripeptide.

Abbreviations: R_{18}, octadecylrhodamine-β-chloride; DM, decyl-β-D-maltoside; DDM, *n*-dodecyl-β-D-maltoside; $C_{10}E_8$, octaethylene glycol monodecyl ether; $C_{12}E_8$, octaethylene glycol monododecyl ether; TX_{100}, poly(ethylene glycol)p-isooctylphenyl ether; CSPD, disodium 3-(4-methoxyspiro{1,2,-dioxyetane-3,2 (5 chloro)tricyclo-[3.3.1.1.]decan}-4-yl)phenyl phosphate; MPB, 3-(N-maleimidylpropionyl)biocytin; AMdiS, 4-acetamido-4-maleimidylstilbene-2,2-disulphonic acid. PC, phosphatidylcholine.

Introduction

The di-tripeptide transport protein DtpT of *Lactococcus lactis* belongs to an important family of proteins that is found in prokarya and eukarya. Other members of the family (Steiner *et al.* 1995) include the peptide transport proteins of *Saccharomyces cerevisiae* (PTR2) (Perry *et al.* 1994), *Candida albicans* (CaPTR2) (Basrai *et al.* 1995), *Arabidopsis thaliana* (AtPTR2A and AtPTR2B) (Steiner *et al*, 1994, Song *et al.* 1996), rabbit intestine (rbPEPT1) (Fei *et al.* 1994), and human intestine (hPEPT1) (Liang *et al.* 1995).

Unlike the majority of bacterial peptide transport systems, the DtpT protein of *L. lactis* catalyses the uptake of di- and tripeptides in symport with (a) proton(s). Although the similarity between the bacterial DtpT and the eukaryotic homologues is low, that is 20–25% identity at the amino acid level, the proteins most likely have a similar molecular architecture. Membrane topology studies have revealed the location of the individual transmembrane segments in the DtpT protein and indicated that the protein spans the membrane 12 times with the amino- and carboxyl-termini located on the inside (Hagting *et al.* 1996).

A major obstacle to characterizing a membrane protein is the difficulty of purifying and maintaining it in its functional form. As a next step towards the further characterization of the DtpT protein, an improved procedure was developed for the purification of the DtpT protein, which takes advantage of the fact that DtpT is poorly solubilized in the presence of $C_{10}E_8$. Following pre-extraction of membrane vesicles with $C_{10}E_8$, the membranes are approximately 10-fold enriched in DtpT, which allows one to fully purify the protein in a single affinity chromatography step. The protein DtpT was functionally reconstituted into detergent-destabilized preformed liposomes. The parameters, which could affect the recovery of activity, were analysed during each step of purification and membrane reconstitution. These include: (i) the efficiency of reconstitution; (ii) losses of lipid; (iii) ion permeability of the proteoliposomes; (iv) lipid to protein ratio in the proteoliposomes; and (v) orientation of the protein in the proteoliposomes.

Results

Purification of DtpT

To obtain DtpT protein with a higher purity than described previously (Hagting *et al.* 1997b) and yet keep the procedure simple, membrane vesicles were pre-extracted with varying concentrations of different detergents prior to the solubilization step. Figure 1a shows the SDS–PAGE analysis of the membranes extracted with different detergents. Clearly, DDM and TX_{100} nearly completely solubilized the membranes, including DtpT, and only a small fraction of membrane proteins was found in the pellet when these detergents were used at or above concentrations of 0.8% (w/v) and 1% (w/v), respectively. A similar observation was made when membranes were treated with $C_{10}E_8$ or $C_{12}E_8$ at final concentration of 1.5 or 2%. However, treatment of the membranes with about 1% of $C_{10}E_8$ or $C_{12}E_8$ resulted in a drastic removal of membrane proteins other than DtpT. Western analysis revealed that

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with 1.0–1.1% of $C_{10}E_8$, up to 85% of DtpT remained in the membrane fraction. Subsequent treatment of the pre-extracted membranes with 1% DDM solubilized nearly all of the remaining proteins. The DDM-extract was applied to a Ni-NTA resin column from which the DtpT protein could be purified as described under experimental procedures. Figure 1b shows that pre-extraction of membranes with $C_{10}E_8$ followed by Ni-NTA affinity chromatography yielded DtpT at a purity that was comparable to DEAE ion-exchange chromatography followed by Ni-NTA purification of a crude-membrane extract (no pre-extraction with $C_{10}E_8$). Since the $C_{10}E_8$ pre-extraction procedure in combination with solubilization of the membrane with DDM and purification on Ni-NTA was quicker and as good as purification by DEAE/Ni-NTA chromatography, this procedure was used throughout the further studies. The procedure yielded approximately 36 mg of protein with a purity >97% from 1 g of membrane vesicles (table 1).

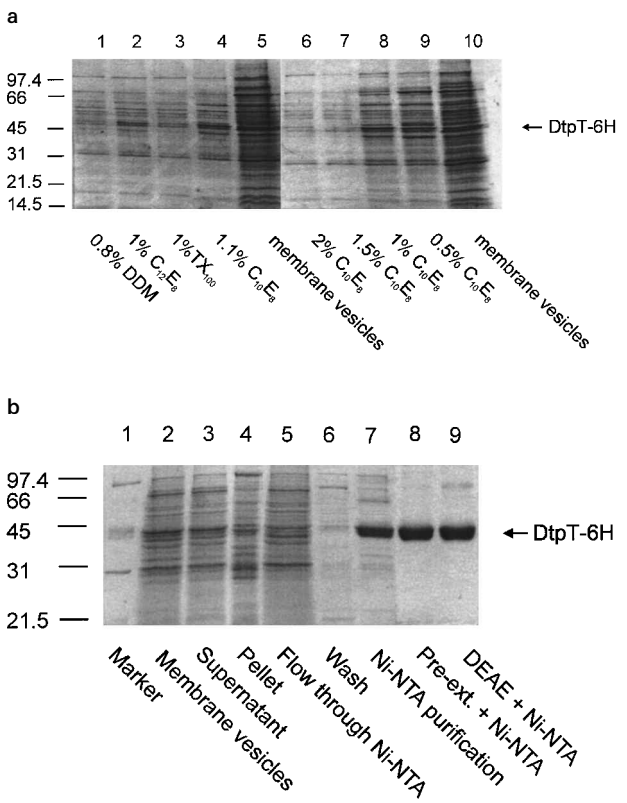

Figure 1. SDS–PAGE analysis of membrane vesicles pre-extracted with different detergents (a) and of the purification of DtpT (b). (a) Membrane vesicles were extracted with different detergents for 30 min at 4°C. Subsequently, the extracts were centrifuged and the pellet fractions were dissolved in buffer for solubilization by 1.0% (w/v) DDM and analysis by SDS–PAGE (12%). Lane 1: 0.8% (w/v) DDM; lane 2: 1% (w/v) $C_{12}E_8$; lane 3: 1% (w/v) TX_{100}; lane 4: 1.1% (w/v) $C_{10}E_8$; lane 5: membranes vesicles of AG300/pGKHT; lane 6: 2% (w/v) $C_{10}E_8$; lane 7: 1.5% (w/v) $C_{10}E_8$; lane 8: 1% (w/v) $C_{10}E_8$; lane 9: 0.5% (w/v) $C_{10}E_8$; lane 10: membrane vesicles of *L. lactis* AG300/pGKHT. (b) Lane 1: molecular weight markers; lane 2: membrane vesicles of *L. lactis* AG300/pGKHT; lane 3: supernatant after solubilization with 1% (w/v) DDM; lane 4: 10-fold concentrated pellet after solubilization with DDM; lane 5: flow-through from Ni-NTA column; lane 6: wash of Ni-NTA column with 4 ml buffer A plus 15 mM; lane 7: elution fraction with 200 mM imidazole; lane 8: sample obtained after pre-extraction of membrane vesicles with $C_{10}E_8$ followed by DDM-solubilization and purification by Ni-NTA chromatography; and lane 9: sample obtained after DDM-solubilization and purification by DEAE sephacel chromatography followed by Ni-NTA.

Detergent-mediated membrane reconstitution of DtpT

To study the ability of different detergents to mediate membrane reconstitution of DtpT, preformed liposomes were treated with varying amounts of detergent (figure 2). The amounts of detergent used for the membrane reconstitution corresponded to the onset of solubilization (R_{sat}) or full solubilization of the preformed liposomes (R_{sol}) (Rigaud *et al.* 1995, Knol *et al.* 1996). The stages of destabilization of the preformed liposomes were determined from turbidity measurements; typical experiments showing the concentration dependence of the solubilization of the liposomes by TX_{100}, $C_{12}E_8$, DM, or DDM are shown in figure 2. Following destabilization of the liposomes with detergents, DtpT was added and further treatments were done as described under experimental procedures. In order to be sure that the onset of solubilization or the point of full solubilization was reached, the detergent concentrations corresponding to R_{sat} or R_{sol}, used in the membrane reconstitution experiments of DtpT, were chosen to be about 10% higher than shown by the arrows in figure. 2. Table 2 shows that TX_{100}, $C_{12}E_8$ and DM were capable of membrane reconstituting DtpT with relatively high efficiency as judged from the Δp-driven uptake measurements. DDM-mediated reconstitution led to significantly lower transport activities. These differences could have several reasons such as: (i) the efficiency of reconstitution could be different; (ii) the various detergents could effect the native state of DtpT differently, even though the protein is relatively shortly exposed to the amphiphiles; (iii) the residual detergent in the proteoliposomes could be different, which is likely to affect the electrochemical proton gradient driven uptake assay; (iv) the final lipid-to-protein ratio in the proteoliposomes could be different for the various detergents. Since DDM- and TX_{100}-mediated reconstitutions represented the extreme cases, and macromolecular structures of liposomes treated with these detergents have been studied in great detail (Knol *et al.* 1998), further work was focused on these amphiphiles.

Table 1. Protein recovery for each step in the solubilization and purification of DtpT from *Lactococcus lactis*.

Purification steps	Protein (mg)	DtpT (% of total protein)	Yield (% of DtpT)
Crude membranes	10	5	100
$C_{10}E_8$-extracted vesicles	1.06	40	85
DDM-solubilized vesicles	1.05	40	84
Ni-NTA purified DtpT	0.37	>97	72

Crude membranes correspond to inside-out membrane vesicles. The percentage of DtpT in each sample was estimated by densitometry of SDS–PAGE. The yield of DtpT was calculated relative to the amount of DtpT present in the crude membranes (100%).

Efficiency of reconstitution

To obtain information about the efficiency of DtpT reconstitution, the proteoliposomes were subjected to sucrose density gradient centrifugation. All DtpT protein was found to be associated with the lipid (liposomes) fractions, both in case of DDM- (figure 3a) or TX_{100}-mediated membrane reconstitution (figure 3b). Equilibrium density centrifugation revealed two populations of liposomes, which is most

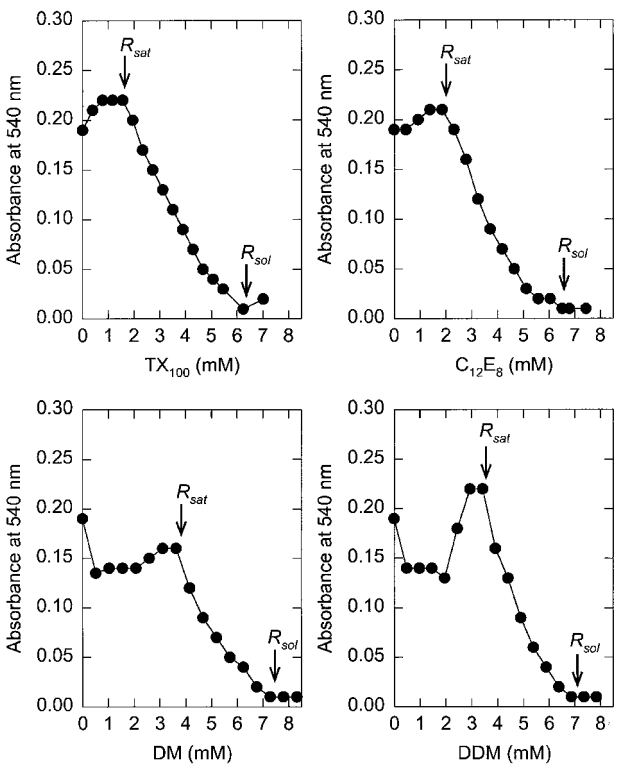

Figure 2. Titration of preformed liposomes with different detergents. The liposomes (4 mg/ml) were titrated with different concentrations of TX_{100}, $C_{12}E_8$, DM or DDM. To follow the solubilization of the liposomes, the turbidity (absorbance at 540 nm) of the suspension was determined after equilibration of the lipid/detergent mixtures at a temperature of 20°C.

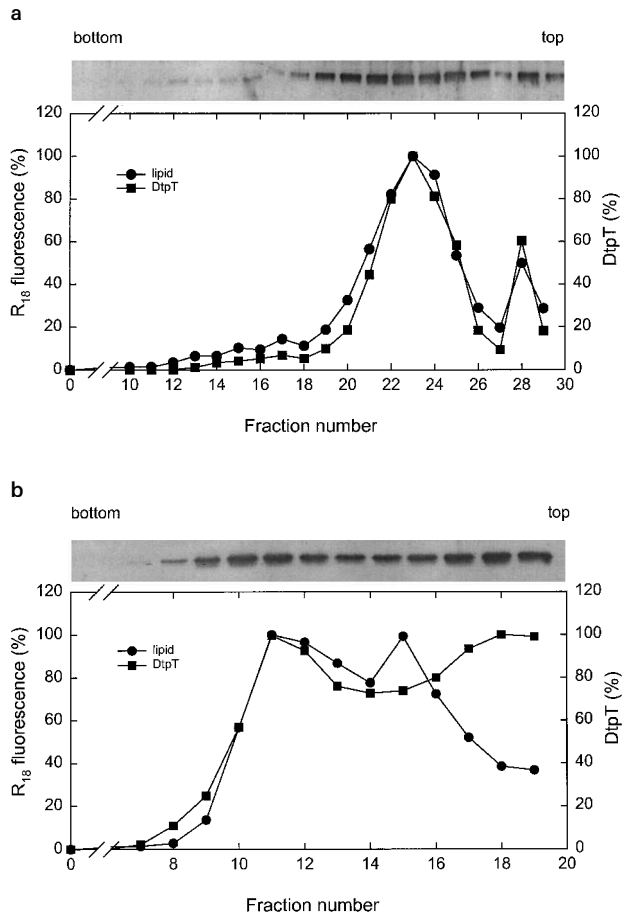

Figure 3. Sucrose gradient centrifugation of proteoliposomes obtained after DDM- and TX_{100}-mediated reconstitution. (a) Preformed liposomes (4 mg/ml) were equilibrated with 3.8 mM DDM (R_{sat}) prior to the addition of DtpT (40 mg/ml, final concentration). After centrifugation, the gradients were fractionated and assayed for the presence of lipid (R_{18} fluorescence) (●) and DtpT (■). The amount of DtpT was determined after immunoblotting and densitometric scanning; the blot is shown at the top of the figure. (b) The experimental condition is the same as above, except preformed liposomes (4 mg/ml) were equilibrated with 1.8 mm TX_{100} (R_{sat}) prior to the addition of DtpT (40 mg/ml, final concentration) and less fractions were collected for analysis.

Table 2. Initial rate of Tyr-Ala uptake in proteoliposomes prepared via TX_{100}-, $C_{12}E_8$-, DM- or DDM-mediated reconstitution.

Detergent	Concentration (mM)	Final lipid-to-protein ratio (w/w)	Δp-driven uptake of Tyr-Ala (nmol/min ×mg of protein)
TX_{100}	1.8 (R_{sat})	102±5	120±8
TX_{100}	6.8 (R_{sol})	99±5	95±7
$C_{12}E_8$	2.3 (R_{sat})	101±5	105±14
$C_{12}E_8$	7.2 (R_{sol})	93±4	78±11
DM	4.1 (R_{sat})	100±3	131±17
DM	8.0 (R_{sol})	99±4	111±12
DDM	3.8 (R_{sat})	94±3	74±9
DDM	7.6 (R_{sol})	76±9	21±4

R_{sat} and R_{sol} correspond to the onset and full solubilization, respectively, of performed liposomes (4 mg/ml of lipid) as determined by A_{540} measurements. The initial lipid-to-protein ratio was 100; the final lipid-to-protein ratios were estimated from the amounts of DtpT and lipid after reconstitution. The lipid was estimated from R_{18} fluorescence and phosphatidylcholine measurements. The protein concentration of the proteoliposomes was estimated by densitometry of SDS–PAGE, using known concentrations of DtpT as standard; no significant losses of DtpT were observed for the different reconstitution trials. The concentration of [^{125}I]-Tyr-Ala in the Δp-driven uptake assay was 10 μM.

clearly observed with DDM. The fraction with the highest density contained 80–90% of DtpT and mainly consisted of unilamellar vesicles. The remaining 10–20% of DtpT was associated with liposomes that were most likely multilamellar, because the peak at lower density disappeared upon a single freeze–thaw-extrusion step (data not shown). Preliminary analysis of the proteoliposomes by cryo-transmission electron microscopy (cryo-TEM) confirms that the fraction of multilamellar (proteo)liposomes decreases after freeze–thaw-extrusion (Knol, Sjollema and Poolman, unpublished data). As all the protein was associated with liposomes, it seems unlikely that the lower activity of DDM-mediated reconstitution (as compared to the other detergents) was due to a lower efficiency of reconstitution.

Effect of detergents on pH gradient in proteoliposomes

The low Critical Micellar Concentration (CMC) of DDM and other detergents could result in incomplete removal of these amphiphilic molecules from the membranes, even after prolonged incubation with polystyrene beads. Residual detergent in the proteoliposomes could affect the pH gradient that is part of the driving force for the uptake of peptides. Therefore, the maintenance of an artificially imposed proton gradient was determined in proteoliposomes prepared via $C_{10}E_8$-, $C_{12}E_8$-, DM-, DDM- or TX_{100}-mediated reconstitution and subsequently exposed to a freeze–thaw-extrusion cycle. Under the experimental conditions, both a pH gradient (ΔpH) and a membrane potential ($\Delta\Psi$) were generated, but only the (pH measurements are presented here. The observed pH gradient was 1.3 pH units, which is somewhat less (0.4 pH units) than can be expected by diluting the proteoliposomes loaded with potassium acetate 50-fold into buffer containing a membrane impermeable anion (sodium Pipes). It turned out that the pH gradient was more stable in the proteoliposomes prepared by $C_{10}E_8$-, DM-, and TX_{100}-mediated reconstitution than those prepared via $C_{12}E_8$ or DDM (figure 4). These data correlate with the differences in Δp-driven uptake activities in the proteoliposomes (table 2). However, the differences are relatively small under conditions of initial rate measurements and do not explain the variations in transport activities. Overall, the data indicate that the proteoliposomes are well-sealed and are able to maintain a $\Delta\Psi$ (data not shown) and ΔpH for prolonged periods of time.

Effect of lipid-to-protein ratios on peptide uptake

Peptide uptake in proteoliposomes formed at varying lipid-to-protein ratios is shown in figure 5. The specific transport activity was highest at lipid-to-protein ratios of 100 and 200 (w/w), whereas no peptide uptake could be detected when the lipid-to-protein ratio was decreased to 10. Although the initial lipid-to-protein ratio is known, the final ratio could be different in the proteoliposomes due to losses of lipid or protein in the reconstitution process. The losses of DtpT protein were negligible for the different detergents (data not shown). The loss of lipid, following reconstitution mediated by DM, DDM, $C_{12}E_8$, or TX_{100} was estimated from the R_{18} and phosphatidylcholine contents of the proteoliposomes. Table 2 shows that the final lipid-to-protein ratios were close to 100 when reconstitution was mediated by TX_{100}, $C_{12}E_8$, DM,

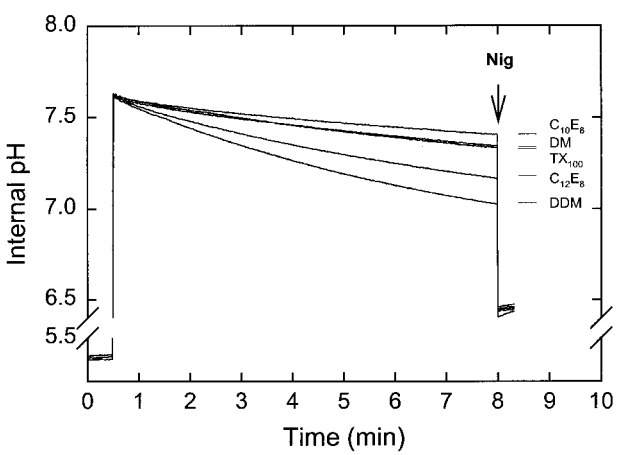

Figure 4. Effect of detergents on the maintenance of the pH gradient in proteoliposomes. Proteoliposomes were formed following DDM-, DM-, $C_{12}E_8$-, $C_{10}E_8$- or TX_{100}-mediated reconstitution of DtpT (see legend to table 2; except that 0.5 mM of pyranine was entrapped). The proteoliposomes were equilibrated with 20 mM potassium phosphate, pH 6.5, 100 mM potassium-acetate plus 2 mM $MgSO_4$. Following centrifugation, the proteoliposomes were resuspended to a final concentration of approximately 1 mg of DtpT/ml. The pH gradient was formed by diluting 40 μl of sample into 2 ml of 120 mM sodium-Pipes, pH 6.5, 2 mM $MgSO_4$, plus 0.5 μM valinomycin. The internal pH was estimated from the changes in pyranine fluorescence.

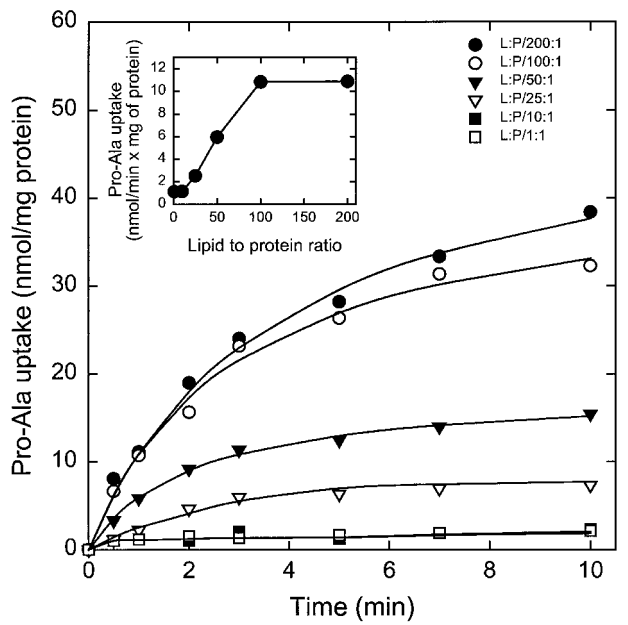

Figure 5. Effect of lipid to protein ratio on Pro-Ala uptake. The DtpT protein was reconstituted at the indicated lipid to protein ratios using 4.1 mM DM (onset of solubilization, R_{sat}). L-Pro-L-[^{14}C]-Ala uptake was assayed as described in materials and methods. The inset shows the dependence of the initial rate of uptake on the lipid to protein ratio.

irrespective of whether the detergents were used at R_{sat} or R_{sol}. Some losses of lipid were observed when DDM was used at R_{sol} to mediate the reconstitution, and the final lipid-to-protein ratio dropped to 76 ± 9%.

Orientation of DtpT in the proteoliposomes

To determine the orientation of DtpT in the proteoliposomes, the single cysteine mutants T360C and V462C were reconstituted, and the accessibility of the cysteines was determined following treatment of the proteoliposomes with the membrane impermeable sulphydryl reagent AMdiS. Subsequent treatment with MPB will only label those cysteines that are not protected by AMdiS, that is those that are at the inner surface of the membrane (or buried inside the protein). Based on previous topology studies (Hagting *et al.* 1997a), Cys-360 is located at the outer surface of the membrane and Cys-462 is at the inner surface of the membrane. To ascertain that this topology is correct, DtpT were first labelled in intact cells by MPB with or without pre-treatment with AMdiS. T360C was indeed accessible from the outside (protected by AMdiS), whereas V462C was present at the inside (not protected by AMdiS) (figure 6a).

Similar experiments were carried out with proteoliposomes using DDM- or TX_{100}-reconstituted T360C and V462C (figure 6b). The MPB-labelling of T360C and V462C was partially protected by the membrane impermeable AMdiS when the reconstitution was mediated by DDM. This suggests that a fraction of reconstituted DtpT protein is always accessible for reaction with AMdiS, and this observation is consistent with a random orientation of DtpT in the proteoliposomes. When the reconstitution was mediated by TX_{100} (at a concentration of 1.8 mM, R_{sat}), the V462C mutant was labelled by MPB, irrespective of whether the proteoliposomes were pre-treated with AMdiS. The T360C protein, on the other hand, was almost completely protected from MPB labelling when the proteoliposomes were pre-treated with AMdiS, indicating that the TX_{100}-reconstituted DtpT protein is largely incorporated in the membrane with an inside-in orientation.

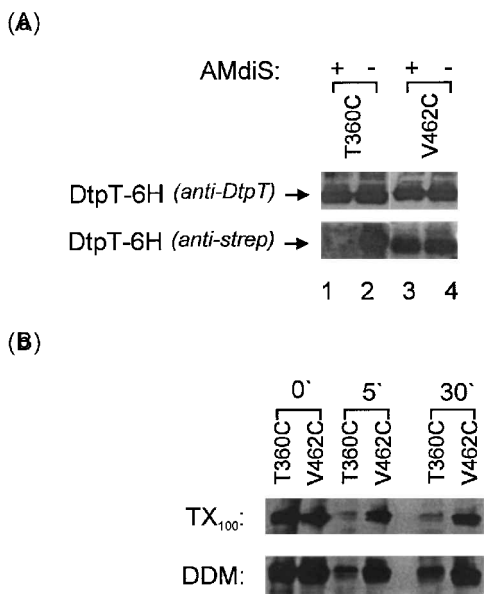

Figure 6. Orientation of DtpT in intact cells and proteoliposomes. (a) *In vivo* labelling: *L. lactis* AG300/pGKHT carrying the *dtpT* alleles T360C or V462C were labelled with MPB with (lanes 1, 3) or without (lanes 2, 4) pre-treatment with AMdiS. The partially purified DtpT proteins were subjected to SDS–PAGE, electroblotted, and visualized by streptavidin-conjugated alkaline phosphatase or anti-DtpT antibodies as indicated. (b) *In vitro* labelling: Purified DtpT (T360C) or DtpT (V462C) was inserted into preformed liposomes that had been destabilized (pre-equilibrated) with 1.8 mM of TX_{100} (R_{sat}) or 3.8 mM DDM (R_{sat}). The proteoliposomes were treated with AMdiS for the indicated time periods, after which the labelling with MPB was performed (see Experimental procedures).

Kinetics of DtpT-mediated transport in proteoliposomes prepared via TX_{100}- and DDM-mediated reconstitution

To determine whether or not the differences in activity in the proteoliposomes prepared with TX_{100} and DDM reflect differences in apparent affinity constants (K_m) and/or maximal rates of transport (V_{max}), Δp-driven uptake was measured at different L-prolyl-L-[^{14}C]alanine concentrations. The data, shown in table 3, indicate that the K_m values are 1.5 ± 0.2 mM and 2.0 ± 0.3 mM for TX_{100}-mediated reconstitution at R_{sat} and R_{sol}, respectively. The corresponding V_{max} values are 1.6 ± 0.2 and 1.2 ± 0.1 μmol/min × mg of protein. The K_m for DDM (R_{sat})-prepared proteoliposomes was 3.1 ± 0.5 mM, whereas the V_{max} was 0.6 ± 0.1 μmol/min × mg of protein. The transport activity in DDM (R_{sol})-prepared proteoliposomes was too low to accurately determine the kinetic parameters of L-prolyl-L-[^{14}C]alanine accurately, but estimates of the V_{max} indicated that it was at most 0.1 μmol/min × mg of protein. The data strongly indicate that the differences in uptake activity in proteoliposomes prepared via TX_{100} and DDM-mediated reconstitution are at the level of the V_{max}, whereas the differences in K_m are small.

Discussion

In this paper, we describe a simple procedure for the purification of the DtpT protein of *Lactococcus lactis*. The method is based on the observation that membrane vesicles of *L. lactis* are very efficiently solubilized by DDM, whereas detergents such as $C_{10}E_8$ and $C_{12}E_8$ are only effective in a limited concentration range (detergent-to-lipid ratio) and at specific ionic conditions. This enabled us to pre-extract the membranes with $C_{10}E_8$ or $C_{12}E_8$, which left the DtpT protein

Table 3. Kinetics of Pro-Ala uptake in proteoliposomes prepared via TX_{100}- or DDM-mediated reconstitution.

Detergent	Concentration (mM)	K_m (mM)	V_{max} (μmol/min × mg of protein)
TX_{100}	1.8 (R_{sat})	1.5 ± 0.2	1.6 ± 0.2
TX_{100}	6.8 (R_{sol})	2.0 ± 0.3	1.2 ± 0.1
DDM	3.8 (R_{sat})	3.1 ± 0.5	0.6 ± 0.1
DDM	7.6 (R_{sol})	*	⩽0.1

Experimental details are the same as described in the legend to table 2, except that Δp-driven uptake of L-Pro-L-[^{14}C]-Ala was determined at different peptide concentrations.
*Activities were too low to obtain an accurate estimate of the K_m.

associated with the membranes whereas many other proteins were solubilized. Subsequent extraction with DDM led to a nearly complete solubilization of the DtpT protein, which could be purified to homogeneity in a single affinity chromatography step.

The membrane reconstitution of an integral membrane protein can often be optimized by following the insertion of the protein into preformed liposomes, destabilized by detergents in the range of R_{sat} to R_{sol}. In this regards, it is worth noting that reconstitution of integral membrane proteins can be regarded as the mirror image of membrane solubilization by detergents (Helenius *et al.* 1975, Rigaud *et al.* 1995). The process of membrane solubilization can be subdivided in three stages (Lichtenberg 1985, Paternostre *et al.* 1988). First, the detergent incorporates into the lipid bilayers (stage I), which results in a peak of the turbidity at the onset of solubilization (R_{sat}). Subsequently, the liposomes disintegrate (stage II) and a transition takes place from bilayer structures to mixed micelles. Finally, at R_{sol}, the liposomes are completely solubilized (stage III) and further increases in detergent concentration only affect the ratio of detergent and lipids in the micelles.

The DtpT reconstitution studies indicate that various detergents are able to mediate the insertion of the DtpT protein into preformed liposomes. In all cases, the majority of DtpT protein was associated with the lipid fraction (liposomes). Consistent with the differences in macromolecular structures formed when TX_{100} and DDM are used to mediate the reconstitution, TX_{100} led to a largely unidirectional and DDM to a random orientation of the DtpT protein in the membrane. By employing cryo-TEM, it has been shown that preformed liposomes titrated with TX_{100} maintained their bilayer structure far beyond R_{sat} (Knol *et al.* 1998), and proteins may insert into these structures in one preferred orientation. With DDM, the vesicular structures are already disrupted at the onset of solubilization and these membrane sheets are converted into long thread-like micelles at higher DDM to lipid ratios (Knol *et al.* 1998, Lambert *et al.* 1998). Proteins are likely to insert into these macromolecular structures from both sides, which leads to a random orientation.

It has previously been shown that LacS inserts into detergent-destabilized liposomes unidirectionally when TX_{100} is used and randomly when DDM is used (Knol *et al.* 1998). The same findings have now been made for the DtpT protein. An important difference concerns the observation that unidirectional reconstitution of LacS corresponds to an inside-out orientation, whereas the DtpT protein is incorporated right-side out. These differences may relate to the 19 kD carboxyl-terminal domain that is present at the cytoplasmic surface of the LacS protein, which gives it a very distinctive hydrophilic surface.

In a previous communication, preliminary evidence was presented about the orientation of DtpT in proteoliposomes prepared via DDM-mediated reconstitution (Hagting *et al.* 1997b). Subsequent work (this study) indicated that one easily reaches the wrong conclusion when the accessibility of cysteines for membrane permeable and impermeable thiol reagents is inferred from a single mutant. It was noted that AMdiS, although clearly membrane impermeable in intact cells, slowly crossed the proteoliposomal membrane. As a result, when incubations were carried out for too long a period of time and/or too high a reagent concentration, labelling was observed even when the cysteine was at the internal surface of the proteoliposomal membrane. In the experimental set-up, this resulted in prevention from labelling by the membrane-permeable MPB. It is, therefore, essential to use more than one cysteine mutant, that is at least one with a unique cysteine in an external loop and one with a cysteine in an internal loop region, and thereby have an internal control for the labelling reactions. The here-described labelling conditions were optimized for Cys-360 (external loop) and Cys-462 (internal loop), and pre-labelling of these mutants with AMdiS was tested at different concentrations and for different time intervals. Subsequent labelling with MPB allowed detection of those proteins that had not reacted with AMdiS.

As shown in table 2, the transport activity of the DtpT protein differed relatively little when, with the exception of DDM, different detergents were used to mediate the reconstitution. The differences between TX_{100}- and DDM-mediated reconstitution seem to reflect differences in maximal uptake activity rather variations in the affinity constants for transport. It was important to determine the K_m values for peptide uptake in the proteoliposomes prepared via TX_{100} and DDM-mediated reconstitution as the orientation of the DtpT protein in the membrane is different for these two conditions. The orientation parameter has been shown to greatly affect the uptake by the LacS protein of *S. thermophilus* as the K_m values are almost two orders of magnitude different for the two opposing directions of transport via this system (Knol *et al.* 1998). Clearly, such a situation does not hold for the DtpT protein.

The extremely low transport activity when DDM is used at R_{sol} to mediate the membrane reconstitution of DtpT may in part relate to the loss of lipid in the reconstitution process as the lipid-to-protein ratio of the proteoliposomes is a critical factor of DtpT activity. Similar observations have been made for the LacS protein, and, in general, DDM is found to yield relatively poor activities and a lower reproducibility when it is used to mediate the membrane reconstitution of solute transport proteins. At the same time, it is stressed that DDM is very effective in keeping membrane (transport) proteins in their native conformation when they are present in the detergent-solubilized state. This implies that in some instances it may be advisable to isolate and purify a protein with the aid of DDM, and, subsequently, to exchange the detergent for another one prior to membrane reconstitution to obtain maximal transport activity.

Experimental procedures

Materials

All peptides were composed of L-amino acids unless otherwise stated. L-[^{14}C]-alanine was obtained from Amersham, UK, Ni-NTA resin from Qiagen, Inc. Germany, L-prolyl-L-[^{14}C]-alanine was synthesized from L-[^{14}C]-alanine and unlabelled N-butyloxycarbonylproline (Boc-Pro) as described previously (Hagting *et al.* 1997b). Decyl-β-D-maltoside (DM), *n*-dodecyl-β-D-maltoside (DDM), octaethylene glycol monodecyl ether ($C_{10}E_8$), octaethylene glycol monododecyl ether ($C_{12}E_8$), N,N-dimethyldodecylamine-N-oxide (DDAO), and 3-[(3-cholamidopropyl)dimethylammoniol]-1-propanesulphonate (CHAPS) were obtained from Fluka, Germany, TX_{100}

was from Boeringher Mannheim, Germany and Bio Beads SM2 from Bio-Rad, USA (20–50 mesh). Total *E. coli* lipids and egg yolk L-α-phosphatidylcholine were obtained from Avanti Polar Lipids, USA; octadecylrhodamine-β-chloride (R_{18}), AMdiS, MPB were from Molecular Probes, Inc., USA Factor Xa was from Biolabs, New England. All other materials were reagent grade and obtained from commercial sources.

Bacterial strains and growth conditions

L. lactis AG300/pGKHT was grown at 29°C in M17 (Difco) or in chemically defined medium (Poolman and Konings 1988) at pH 6.5, and supplemented with 0.5% (w/v) glucose plus erythromycin (5 μg/ml) as described by Hagting *et al.* (1997b).

Isolation of membrane vesicles

Right-side-out and inside-out membrane vesicles of *L. lactis* AG300/pGKHT cells were isolated as described previously (Otto *et al.* 1982, Poolman *et al.* 1983) with some modifications. For the isolation of inside-out membrane vesicles, the cell wall was digested with 10 mg/ml of lysozyme, the cells were broken by a 2-fold passage through a french pressure cell at 20 000 p.s.i., and DNase was added to a final concentration of 0.1 mg/ml. The membrane preparations were stored in liquid nitrogen.

Solubilization

The membranes were solubilized in two successive steps using different detergents. First, membrane proteins other than DtpT were partially removed by treatment of the membrane vesicles with $C_{10}E_8$. Membranes (10 mg of protein/ml) were pre-extracted in 50 mM potassium phosphate, pH 8.0, containing 400 mM sodium chloride, 10% (v/v) glycerol (buffer A) plus varying concentrations of $C_{10}E_8$ as indicated in the legends to figures. The suspensions were incubated for 30 min at 4°C, and solubilized proteins (S_1) were removed by centrifugation (280 000 g, 15 min, 4°C). The pellet (P_1) was resuspended in buffer A plus 1% of DDM. The volume was the same as for the first solubilization step. Following incubation for another 30 min at 4°C, the non-solubilized proteins (P_2) were removed by centrifugation at 280 000 g, 15 min, 4°C. The supernatant (S_2), containing more than 80% of DtpT, was used for further purification of the protein by nickel-chelate affinity chromatography.

Purification

The S_2 extract was mixed and incubated for 30 min at 4°C with Ni-NTA resin (~ 4 mg of DtpT/ml of resin), which was equilibrated with buffer A plus 0.1% DDM (buffer B). The column material was poured into a Bio-Spin Column (Bio-Rad) and washed consequetively with five column volumes of buffer B, buffer B plus 5 mM imidazole, buffer B plus 15 mM imidazole except that the sodium chloride concentration was lowered to 200 mM. The protein was eluted with 50 mM potassium phosphate, pH 6.5, 200 mM sodium chloride, 10% glycerol, 200 mM imidazole plus 0.1% DDM. The purification was carried out at 4°C with sterile solutions. For the DEAE-sephacel purification, the membrane vesicles solubilized with 1% DDM were diluted 10-fold with 50 mM potassium phosphate, pH 7.0, 10% glycerol plus 0.1% DDM and applied to an anion exchange column (DEAE). The eluent was mixed with Ni-NTA resin and purified as described above.

Reconstitution

The purified DtpT protein was reconstituted into preformed liposomes, prepared from acetone/ether washed *E. coli* lipids and L-α-phosphatidylcholine from egg yolk in a ratio of 3:1 (w/w) as described (Knol *et al.* 1996, Poolman and Konings 1988) with some modifications. The acetone/ether washed lipid extract is composed of approximately 70% of phosphatidylethanolamine (PE), 25% of phosphatidylglycerol and 5% of cardiolipin (unpublished results). Purified DtpT was mixed with preformed detergent-destabilized liposomes at a lipid-to-protein ratio of 100:1 (w/w), unless specified otherwise. The mixture was incubated for 20 min at room temperature under gentle agitation. For the removal of detergent, polystyrene beads (Biobeads SM2, Bio-Rad) were added at a wet weight of 60 mg/ml and the samples were incubated for another 1 h at room temperature. Fresh biobeads were added twice and the samples were subsequently incubated at 4°C for 2 h and overnight. Under these conditions, the losses of lipid were minimal (except for DDM) and the biobeads concentration of 60 mg/ml was, therefore, used for all the reconstitution experiments. The obtained proteoliposomes were washed with 50 mM potassium phosphate, pH 7.0, and stored in liquid nitrogen.

Sucrose gradient centrifugation

Discontinuous sucrose gradients were prepared from the following sucrose concentrations made in 10 mM Tricine-HCl, 100 mM KCl plus 1 mM EDTA, pH 8.0: 45% (w/v) (0.4 ml), 35% (0.4 ml), 28% (0.4 ml), 23% (0.4 ml), 18% (0.4 ml), 13% (0.4 ml), 8% (0.4 ml), 3% (0.4 ml). Samples of 0.2 ml (4 mg of lipid corresponding to 0.04 mg of DtpT protein) were layered on the top of the gradients, and centrifuged for 19 h at 145 000 g_{max} in a SW50i rotor at 18°C. Prior to the equilibrium density centrifugation, the proteoliposomes were fluorescently labelled with octadecylrhodamine-β-chloride (R_{18}) essentially as described (Hoekstra *et al.* 1984). R_{18} (50 nmol in 5 μl ethanol) was added under extensive vortexing to a 1 ml suspension of proteoliposomes. The mixture was incubated in the dark for 1 h at room temperature and non-incorporated R_{18} was removed by washing the membranes three times with 50 mM potassium phosphate, pH 7.0 (280 000 g for 15 min). The R_{18} fluorescence was measured in the presence of 1% (v/v) TX_{100} at excitation and emission wavelengths of 560 nm and 590 nm, respectively.

Transport assays

Artificially imposed ion-diffusion potentials were generated as described (Foucaud and Poolman 1992). Proteoliposomes were resuspended in 20 mM potassium phosphate, pH 6.5, 100 mM potassium acetate plus 2 mM $MgSO_4$ and frozen in 0.5 ml aliquots in liquid nitrogen. After thawing the samples at room temperature, the proteoliposomes were extruded 11 times through a 400 nm polycarbonate filter to obtain unilamellar liposomes of relatively homogenous size (Knol *et al.* 1998). Subsequently, aliquots of concentrated membrane suspensions (1 mg of protein/ml) were diluted 50-fold into 120 mM NaPipes, pH 6.5, 2 mM $MgSO_4$, containing 0.5 μM valinomycin plus L-Pro-L-[^{14}C]-Ala at varying concentrations (see legends to figures). The uptake was assayed for different time intervals at 30°C, after which the reaction was stopped by diluting the mixture with 2 ml of ice-cold 0.1 M LiCl. The proteoliposomes were collected on 0.45 μm cellulose nitrate filters and washed once more with 2 ml of the LiCl solution. The counterflow assays were performed as described (Knol *et al.* 1996) except that the proteoliposomes were loaded with 5 mM Tyr-Ala. The reaction was stopped as mentioned above.

Measurement of proton gradient in proteoliposomes

The internal pH of the proteoliposomes was estimated from the fluorescence of the pH indicator pyranine (excitation and emission wavelengths of 450 and 508 nm, respectively). Pyranine (0.5 mM, final concentration) was entrapped in the proteoliposomes by freeze/thaw/extrusion, as described previously (Driessen and Konings 1993). External pyranine was removed by gel filtration on Sephadex G25. The fluorescence units were converted into pH units, after treatment of the proteoliposomes with nigericin (internal and external pH become equal), subsequent titration with small aliquots (1 μl) of 2 M NaOH, and recording of the fluorescence and pH values in parallel.

Orientation of membrane-reconstituted DtpT

Proteoliposomes (0.1 mg/ml of protein) were treated with 100 μM AMdiS or untreated for 5–30 min at 30°C. Subsequently, the membranes were washed twice with 50 mM potassium phosphate, pH 8.0, resuspended at 0.1 mg/ml of protein, and incubated for 30 min at 30°C with 200 μM MPB. The reactions were stopped by the addition of 10 mM dithiothreitol and analysed by Western blotting and detection with streptavidin/alkaline phosphatase and CSPDTM as a substrate. The *in vivo* labelling of DtpT by AMdiS and MPB was carried out as described previously (Hagting *et al.* 1997b).

Protein determination

The Lowry assay (Lowry *et al.* 1951) was used to determine the protein concentration of membrane vesicles or solubilized protein. In order to estimate the amount of reconstituted DtpT protein (associated with the lipid fraction), the proteoliposomes were dissolved in SDS-sample buffer and analysed by SDS–PAGE. The amount of DtpT was estimated by densitometry after the staining of the gel; purified DtpT of known concentration was used as standard.

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